Supplementary Materials and Methods

***In vivo* ubiquitination assay**

To purify ubiquitinated Paip2 proteins, HeLa cells were transfected with a plasmid encoding His-Ub (Treier *et al*, 1994). At 24 h post-transfection siRNA-transfected cells were treated with 20 μM MG132 for 6h. Cells were lyzed in buffer A (6M guanidine-HCl, 100 mM Na_2HPO_4/NaH_2PO_4 pH 8.0 and 10 mM imidazole). Lysates were sonicated to reduce the viscosity. After centrifugation, His-tagged ubiquitinated proteins were purified using a TALON metal affinity resin (Clontech), as described previously (Treier *et al*, 1994). Purified proteins were eluted from the resin by adding 2x Laemmli sample buffer supplemented with 0.2 M imidazole, and processed for Western blot analysis with anti-Paip2 antibody as indicated.

***In vitro* ubiquitination assay**

Ubiquitin activating enzyme (E1) and ubiquitin conjugating enzyme (E2), GST and GST-Paip2 were prepared as previously described (Wing and Jain, 1995; Khaleghpour *et al*, 2001a). EDD (E3) was isolated from 24 mg of testis lysate of a 40-day-old rat by immunoprecipitation with 81 μg of immuno-affinity purified anti-EDD antibody (Oughtred *et al*, 2002) bound to 560 μl (50% slurry) of protein A Sepharose beads. The pellet was washed twice with cold 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 0.5% Nonidet P-40 and protease inhibitor cocktail, followed by twice washing with 50 mM Tris-HCl (pH 7.5), 1 mM DTT. The ubiquitination reaction contained 50 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 0.5 mM DTT, E1 (125 nM), E2

(UBC4-1, 500 nM), purified GST protein (4.5 μM) or GST-Paip2 (4.5 μM), AMP-PNP (5'-adenylylimidodiphosphate lithium, 2 mM), ubiquitin aldehyde (5 μM) and as E3, 14% of the pellet containing immunoprecipitated EDD. Following pre-incubation at 37 °C for 5 minutes, His-ubiquitin (5 μM) was added to start the reaction. AMP-PNP, can be used by ubiquitin activating enzyme (which cleaves ATP to AMP and PPi) to support ubiquitin conjugation, but not by the proteasome which cleaves ATP to ADP and Pi (Johnston and Cohen, 1991). We routinely use AMP-PNP in conjugation reactions to prevent degradation of ubiquitinated protein products by any contaminating proteasome in our reagents. Ubiquitin aldehyde is an inhibitor of many deubiquitinating enzymes(Hershko and Rose, 1987) and can therefore prevent the loss of ubiquitinated proteins by such contaminating enzymes. Ubiquitin aldehyde was synthesized as described (Wilkinson et al, 1986). UBC4 family isoforms were used because they have been previously shown to be the most effective in supporting EDD mediated ubiquitination when a large panel of conjugating enzymes were tested (Honda *et al*, 2002). The reaction (120 μl) was incubated at 37 °C for 55 minutes with gentle agitation every 10 minutes. Following centrifugation at 1000 rpm for 1 min to isolate the beads, the supernatant from each reaction was mixed with 30 μl (50% slurry) of glutathione agarose beads (Amersham Bioscience) in 1 ml RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8.0)) at room temperature for 2 hours to isolate the products. The pellets were washed four times with 1ml RIPA buffer, then suspended with 3x Laemmli sample buffer containing 10% 2-mercaptoethanol, boiled for 10 minutes, and processed for Western blot analysis with anti-GST, anti-Paip2, or anti-ubiquitin (Sigma) antibodies as indicated.

PABC expression and purification

The PABC domain of human EDD (residues 2393 to 2452) was cloned into BamHI and EcoRI restriction sites of the pGEX-6P-1 vector (Amersham Pharmacia Biotech, Piscataway, NJ), and the plasmid was transformed into the *E. coli* expression host BL21 Gold Magic (DE3) (Stratagene). The protein was expressed and purified by affinity chromatography to yield the 60 residues of EDD PABC plus a five-residue (Gly-Pro-Leu-Gly-Ser) N-terminal extension. The amino acid composition was confirmed by mass spectrometry. For NMR analysis, EDD PABC was exchanged into NMR buffer (50 mM Tris-HCl (pH 6.8), 100 mM NaCl, 1 mM NaN_3). The final yield of purified protein was 7 mg per liter of M9 culture media. The Paip2 peptide VVKSNLNPNAKEFVPGVKYGNI was synthesized by Fmoc solid-phase peptide synthesis and purified by reverse phase chromatography on a C18 column (Vydac, Hesperia, CA). The composition and purity of the peptide was verified by ion-spray quadrupole mass spectroscopy.

NMR spectroscopy

NMR resonance assignments of the EDD PABC were determined by HNCACB and CBCA(CO)NH experiments (Grzesiek and Bax, 1993) on a ^{13}C,^{15}N-labeled sample with a Bruker Avance600 NMR spectrometer. Assignments of amide resonances in the PABC-Paip2 complex were based on ^{15}N-^{1}H edited NOESY and TOCSY spectra obtained at 600 MHz. All NMR experiments were recorded at 303 K. Peptide NMR titrations were carried by adding peptide into ^{15}N-labeled EDD PABC and the acquisition of ^{15}N-^{1}H

heteronuclear single quantum correlation (HSQC) spectra. The results of two HSQC of the PABC domain from EDD are shown in Supplementary Figure S2.

Isothermal Titration Calorimetry (ITC) measurements

Experiments were carried out on a MicroCal VP-ITC titration calorimeter (MicroCal Inc., Northampton, MA) using the VPViewer software for instrument control and data acquisition. The buffer used for ITC experiments contained 50 mM Tris-HCl (pH 6.8) and 100 mM NaCl. During a titration experiment, samples of EDD PABC fragment were thermostated at the desired temperature in a stirred (310 rpm) reaction cell of 1.4 ml. Fifty nine injections, each of 5 μl volume and 10 s duration with a 5-min interval between injections, were carried out using a 296 μl syringe filled with the peptide solution. Titration experiments were performed with 30-60 μM PABC solution in the cell and 250-550 μM peptide solution in the syringe to ensure a final peptide: PABC molar ratio of 2:1 in the reaction cell. The binding constants and thermodynamic parameters were determined as described earlier (Kozlov *et al*, 2004).

Surface Plasmon Resonance (SPR)-based biosensor analysis

The interaction between the PABC domain of EDD and Paip2 peptide was investigated by surface plasmon resonance using a BIACORE 3000 optical biosensor (Biacore Inc, Piscataway, NJ). Temperature was set at 25°C. The PABC domain was immobilized onto a CM5 biosensor chip surfaces using standard amine coupling chemistry as described previously (Johnsson *et al*, 1991). Injections of Paip2 peptide solutions at various concentrations (7.8×10^{-7} to 10^{-4} M) were performed in triplicate over two PABC surfaces

with different amounts of protein bound (approximately 1300 and 4000 RU coupled) and over a control surface (no PABC domain) to correct for refractive index changes. The experiments were performed as previously described (Kozlov *et al*, 2004). Thermodynamic dissociation constants (K_d) were determined by globally fitting the experimental data derived from the various surfaces according to the following equation: $R_{eq} = R_{maxi} (C / (C + K_d))$, where R_{eq} corresponds to the control corrected plateau values recorded when injecting Paip2 peptide solutions and where R_{maxi} correspond to the saturating amounts of Paip2 (local parameter kept constant for each PABC surface) and K_d which is a global parameter.

References

Hershko A, Rose IA (1987) Ubiquitin-aldehyde: a general inhibitor of ubiquitin-recycling processes. *Proc Natl Acad Sci USA* **84:** 1829-1833

Honda Y, Tojo M, Matsuzaki K, Anan T, Matsumoto M, Ando M, Saya H and Nakao M. 2002. Cooperation of HECT-domain ubiquitin ligase hHYD and DNA topoisomerase II-binding protein for DNA damage response. *J Biol Chem* **277:** 3599-3605

Johnsson B, Lofas S, Lindquist G (1991) Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. *Anal Biochem* **198:** 268-277

Johnston NL, Cohen RE (1991) Uncoupling ubiquitin-protein conjugation from ubiquitin- dependent proteolysis by use of beta, gamma-nonhydrolyzable ATP analogues. *Biochemistry* **30:** 7514-7522

Khaleghpour K, Kahvejian A, De Crescenzo G, Roy G, Svitkin YV, Imataka H, O'Connor-McCourt M, Sonenberg N (2001a) Dual interactions of the translational repressor Paip2 with poly(A) binding protein. *Mol Cell Biol* **21:** 5200–5213

Kozlov G, De Crescenzo G, Lim NS, Siddiqui N, Fantus D, Kahvejian A, Trempe JF, Elias D, Ekiel I, Sonenberg N, O'Connor-McCourt M, Gehring K (2004) Structural basis of ligand recognition by PABC, a highly specific peptide-binding domain found in poly(A)-binding protein and a HECT ubiquitin ligase. *EMBO J* **23:** 272-281

Oughtred R, Bedard N, Adegoke OA, Morales CR, Trasler J, Rajapurohitam V, Wing SS (2002) Characterization of rat100, a 300-kilodalton ubiquitin-protein ligase induced in germ cells of the rat testis and similar to the *Drosophila hyperplastic discs* gene. *Endocrinology* **143:** 3740-3747

Treier M, Staszewski LM, Bohmann D (1994) Ubiquitin-dependent c-Jun degradation in vivo is mediated by the delta domain. *Cell* **78:** 787-798

Wing SS, Jain P (1995) Molecular cloning, expression and characterization of a ubiquitin conjugation enzyme (E2(17)kB) highly expressed in rat testis. *Biochem J* **305:** 125-132

Wilkinson KD, Cox MJ, Mayer AN and Frey T (1986) Synthesis and characterization of ubiquitin ethyl ester, a new substrate for ubiquitin carboxyl-terminal hydrolase. *Biochemistry* **25:** 6644-6649